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Harmen R. Zandbergen, MD, Umesh C. Sharma, MD, PhD, Sudhir Gupta, MD, PhD, Johan W. H. Verjans, MD, Susanne van den Borne, MD, Saraswati Pokharel, MD, PhD, Thomas van Brakel, MD, PhD, Adriaan Duijvestijn, PhD, Nico van Rooijen, PhD, Jos G. Maessen, MD, PhD, Chris Reutelingsperger, PhD, Yigal M. Pinto, MD, PhD, Jagat Narula, MD, PhD, and Leo Hofstra, MD, PhD

Inflammation contributes to the process of ventricular remodeling after acute myocardial injury. To investigate the role of macrophages in the chronic process of cardiac remodeling, they were selectively depleted by intravenous administration of liposomal clodronate in heart failure-prone hypertensive Ren-2 rats from the age of 7 until 13 weeks. Plain liposomes were used for comparison. Liposomal clodronate treatment reduced the number of blood monocytes and decreased the number of macrophages in the myocardium. Compared to plain liposomes, liposomal clodronate treatment rapidly worsened left ventricular ejection

function in hypertensive rats. Liposomal clodronate-treated Ren-2 rat hearts showed areas of myocyte loss with abundant inflammatory cell infiltration, predominantly comprising CD4 positive T lymphocytes. The current study showed that lack of macrophages was associated with earlier development of myocardial dysfunction in hypertensive rats. Modulation of macrophage function may be of value in the evolution of cardiomyopathy.

Keywords: hypertension; inflammation; ventricular remodeling; heart failure

Introduction

Although inflammatory myocardial diseases are well known to induce chronic cardiomyopathic state, sparse inflammatory cell infiltration is commonly

observed in noninflammatory heart muscle disease.^{1,2} It is believed that inflammatory mechanisms contribute to chronic cardiomyopathy by slow and progressive tissue damage and alteration of myocardial architecture or induction of autoimmunity.^{3,4} Macrophages may also contribute to myocardial dysfunction by producing inflammatory and profibrotic mediators.⁵ Accumulating evidence on the pathophysiological consequences of sustained expression of macrophage-based proinflammatory mediators in preclinical and clinical heart failure (HF) models has led to several clinical trials that have used targeted approaches to neutralize tumor necrosis factor (TNF) in patients with moderate to advanced HF.^{6,7} However, these targeted approaches have not yielded the anticipated results. In the current

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study, we investigated the role of macrophages in evolution of ventricular dysfunction in hypertensive homozygous Ren-2 rats. These animals are known to demonstrate rapid progression from hypertension to compensated left ventricular hypertrophy and HF. Male homozygous transgenic rats carrying 2 copies of mouse renin-2 genes (Ren-2 rats) exhibit moderate hypertension, myocardial hypertrophy, and excessive fibrosis associated with overproduction of angiotensin-II (Ang-II). These pathological changes can begin as early as 6 weeks of age and 50% of these rats progress to HF by 12 to 14 weeks, while the other half remain compensated at least till 17 weeks. The failing Ren-2 rat hearts reveal abundant macrophage infiltration. We hypothesized that antagonizing macrophage influence in the heart by a depletion strategy would result in a delay in the development of end-stage hypertensive cardiomyopathy and HF. For this purpose, we injected liposomal clodronate (LC) intravenously in homozygous Ren-2 rats, for selective elimination of macrophages.^{8,9} Liposomal clodronate is phagocytosed by circulating monocytes. Free clodronate does not cross cell membranes and has an extremely short half-life in the circulation. Liposomal clodronate is removed from the circulation by the renal system and has no major adverse effects on cell types that do not phagocytose the liposomes.⁸⁻¹⁰

Methods

Experimental Design

Male, 6-week-old homozygous Ren-2 and Sprague Dawley (SD) rats were obtained from M&B (Bomholtvej, Denmark). Clodronate was a gift from Roche Diagnostics GmbH, Mannheim, Germany. Liposomal clodronate and plain liposomes (PL) were prepared as described previously.⁹ Under light isoflurane anesthesia, 10 male homozygous Ren-2 rats received LC (4 μ L/gram body weight) through the tail vein, every week for 7 weeks. As a control for the liposomally encapsulated clodronate, liposomes filled with phosphate buffer saline (PBS) were injected in 7 Ren-2 rats. In addition, 4 healthy SD rats received LC and 4 age-matched SD rats received PL. Rats were euthanized at 13 weeks. In all experimental groups, blood pressure was measured by tail-cuff method, and echocardiography was performed at 0, 14, 28, and 42 days of LC or PL treatment in rats sedated with 2% isoflurane. Standard views were obtained in 2-dimensional as well as M-mode with a

12-MHz transducer (Sonos 5500, Hewlett Packard, Netherlands) with approximately 220 frames per recording and the data analysis was performed by a blinded observer. The Institutional Animal Care and Use Committee approved the procedure for care and treatment of animals.

Peripheral Blood Monocytes and CD4⁺ and CD8⁺ T cells

Peripheral blood monocytes and CD4⁺ and CD8⁺ T cells were analyzed in Ren-2 rats at 2 and 7 days after intravenous infusion of LC or PL with specific antibodies by flow cytometry. In brief, anticoagulated blood (200 μ L) was incubated for 30 minutes (4°C) with mouse anti-rat R-phycoerythrin (RPE)-conjugated anti-CD4, CD8, and CD68 antibodies (Serotec, Düsseldorf, Germany) and isotype controls. All analyses were performed separately. Fluorescence-activated cell sorting (FACS) lysing solution (1:20 dilution) was added for 15 minutes. The residual cells were washed (\times 1500 rpm, 5 minutes, 4°C) in FACS medium (PBS, 1% bovine serum albumin [BSA], 0.02% sodium azide) and suspended for flow cytometry. Monocytes and lymphocytes were identified according to their relative size, side scattering, and fluorescence.

Immunohistochemical Localization of Macrophages and T Lymphocytes in Myocardium

Immunohistochemistry was performed on cryofixed myocardial sections. After drying the sections for 15 minutes, ice-cold methanol (100%) was added for 10 minutes; sections were washed 3 times with PBS and blocked with 5% horse serum for 60 minutes. Anti-CD3, -CD4, -CD8, and -CD68 (Serotec) and anti-annexin A5 (BioVision, Uithoorn, The Netherlands) antibodies (1:250 in 0.1% BSA in PBS) were applied and incubated overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were then used for 4 hours at room temperature followed by horseradish peroxidase-labeled streptavidin for 60 minutes. After washing 3 times for 5 minutes in PBS, 3,3'-diaminobenzidine tetrahydrochloride reagent was applied to visualize the reaction.

Statistical Analyses

Data are presented as mean \pm SD. The comparisons were made by unpaired *t* test. For multiple

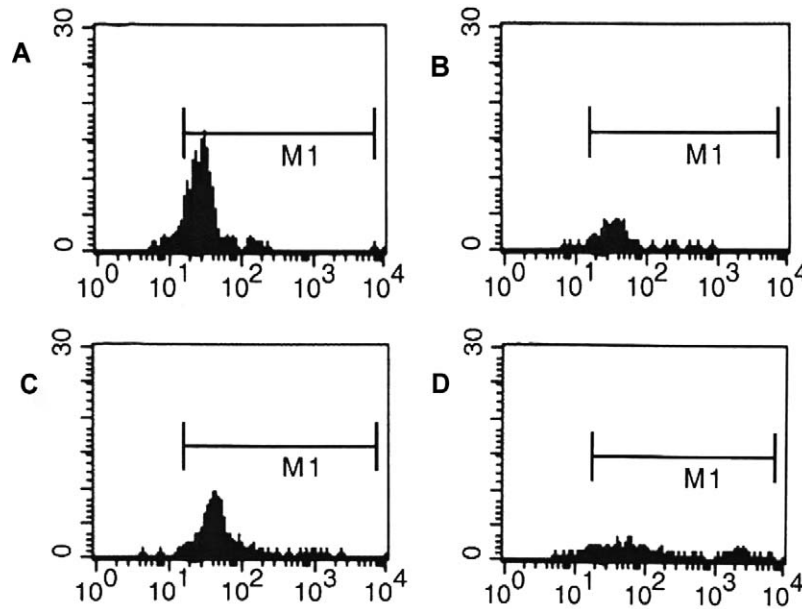


Figure 1. Flow cytometric analysis of blood monocyte population after the infusion of LC or PL in Ren-2 rats. We injected PL or LC in Ren-2 rats and collected the blood samples after 48 hours. Height of the histogram represents monocyte population. A, FACS analysis of CD68 positive cells in the blood in PL-treated Ren-2 rats. B, Isotype control for panel A. C, CD68 positive cells in LC-treated Ren-2 rats. D, Isotype control for panel C. FACS = fluorescence-activated cell sorting; LC = liposomal clodronate; PL = plain liposomes.

comparisons, 1-way analysis of variance (ANOVA) in combination with a Dunnett's post hoc analysis was made. Analyses were performed by the use of the statistical package SPSS 10.0. Probability value of $P < .05$ was considered to be statistically significant.

Results

Peripheral Blood Monocyte Count and the Interstitial Macrophage Infiltration

Baseline peripheral blood monocyte proportion in PL-treated Ren-2 rats was $2.28\% \pm 0.15\%$ (Figure 1A and B). Two days after LC injection in Ren-2 rats, the number of monocytes sharply reduced to $0.81\% \pm 0.21\%$ (Figure 1C and D), whereas CD4 and CD8 count remained unchanged (CD4: LC, $43.6\% \pm 3.5\%$ vs. PL, 45 ± 4.6 ; CD8: LC, $5\% \pm 2.5\%$ vs. PL, 4.8 ± 1.9 , $P = \text{NS}$; Table 1). However, the monocyte count returned to baseline levels (1.8 ± 0.38) by 7 days. Presence of macrophages in the hearts was shown by CD68-specific monoclonal antibody. In the Ren-2 rats that received PL, macrophages were frequently observed (Table 2). However, LC-treated Ren-2 rats showed scattered cells identical to macrophage morphology; the macrophages also

Table 1. Monocyte, CD4, and CD8 Count of LC- and PL-treated Rats (%)^a

	Ren-2-LC	Ren-2-PL
Monocytes	0.81 ± 0.21	2.28 ± 0.15
T-helper cells (CD4)	43.6 ± 3.5	45 ± 4.6
Cytotoxic T cells (CD8)	5 ± 2.5	4.8 ± 1.9

Abbreviations: LC, liposomal clodronate; PBS, phosphate buffer saline; PL, PBS liposomes.

^a $P = \text{NS}$.

stained positively for annexin A5, suggesting LC-induced apoptotic changes in these cells (Figure 2A). Annexin A5 positivity was not seen in the PL-treated Ren-2 rats (Figure 2B) and control rats with LC (Figure 2C) and PL (Figure 2D) treatment.

Evolution of Cardiac Function in Monocyte-depleted Ren-2 Rats

The Ren-2 rats that received LC from 7 to 13 weeks of age demonstrated significant deterioration of myocardial function (ejection fraction (EF) %, baseline, 65.2 ± 4.1 ; 6 weeks, 49 ± 5.4 , $P < .05$). However, Ren-2 rats that received PL showed only a mild and no decrease in cardiac performance (EF%, baseline,

Table 2. Morphometric Data to Compare the Cellular Infiltration and Apoptotic Changes in the Myocardium of LC- or PL-treated Rats

	Cell counts/HPF			
	Ren-2-LC (N = 7)	Ren-2-PL (N = 7)	SD-LC (N = 4)	SD-PL (N = 4)
Mononuclear cells (HE-staining)	89.3 ± 26 ^a	7 ± 2.4	13.8 ± 2.1	8.5 ± 1
Macrophages (CD68)	1.8 ± 0.31	13.8 ± 3.3 ^b	0.66 ± 0.4	2.5 ± 0.7
T-lymphocytes (CD3)	49 ± 13.3 ^a	3.3 ± 0.68	4.8 ± 1.1	4.3 ± 0.8
T-helper cells (CD4)	33.8 ± 13.7 ^a	1.8 ± 0.31	3.5 ± 0.7	3.1 ± 0.8
Cytotoxic T-cells (CD8)	2.8 ± 0.4	2.3 ± 0.50	2.1 ± 0.4	2.3 ± 0.51
Apoptotic cells (Annexin A5)	10.5 ± 4.2 ^a	2.1 ± 0.66	3.1 ± 0.7	1.1 ± 0.4

Abbreviations: HE, hematoxylin-eosin; HPF, high power field; LC, liposomal clodronate; PBS, phosphate buffer saline; PL, PBS liposomes; SD, Sprague Dawley.

^aP < .05 versus Ren-2-PL, SD-LC, and SD-PL.

^bP < .05 versus Ren-2-LC, SD-LC, and SD-PL.

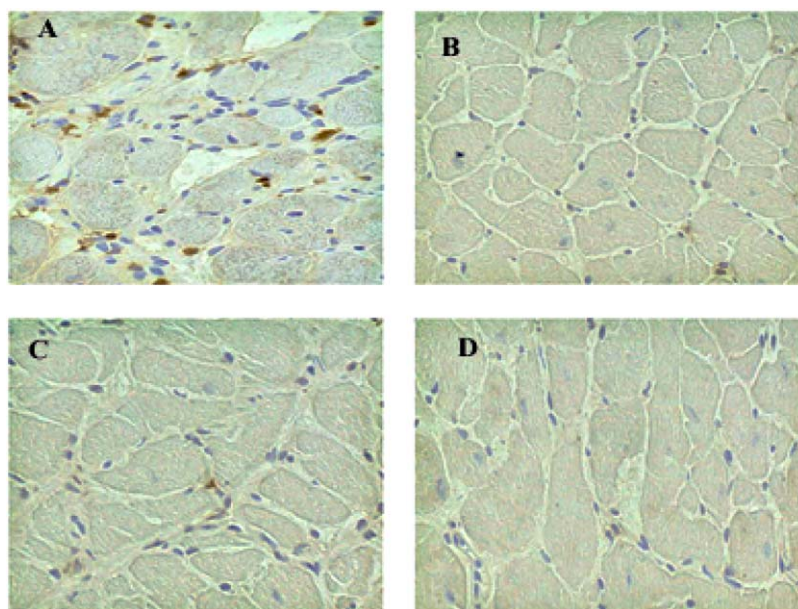


Figure 2. Demonstration of apoptosis-specific changes by annexin A5 positivity. A, Anti-annexin A5 staining showing apoptosis of macrophages in LC-treated Ren-2 rat hearts. B-D, Anti-annexin A5 staining in PL-treated Ren-2 rats, LC-treated SD rats, and PL-treated SD rats, respectively. LC = liposomal clodronate; PL = plain liposomes; SD = Sprague Dawley.

67 ± 3.1; 6 weeks, 62 ± 5.3). The control rats did not show any significant alteration in cardiac performance, regardless of LC (EF%, baseline, 64 ± 4; 6 weeks, 63 ± 3.8) or PL (EF%, baseline, 65.5 ± 1.9; 6 weeks, 66.4 ± 1.0) administration (Table 3). This suggests that persistent macrophage depletion in hypertensive hearts (and normally with abundant macrophage infiltration) accelerates functional deterioration. There was no significant difference in body weight between the 4 experimental groups. Ren-2 rats were hypertensive and had significantly increased heart weight to body weight ratio compared to

controls. However, these parameters were not significantly different between LC- and PL-treated rats (Table 4).

Histological Consequences of Macrophage Depletion

The Ren-2 rats that received LC treatment for 6 weeks showed areas of cardiomyocyte damage (Figure 3A), which was not discernible in control groups (Figure 3B-D). Although there was a marked decrease in number of macrophages, abundant focal

Table 3. Baseline, 2-, 4-, and 6-Week Echocardiographic Parameters in LC- or PL-treated Ren-2 Rats and SD Controls

LV Functional Parameters	Time Point	Ren-2-LC (N = 10)	Ren-2-PL (N = 7)	SD-LC (N = 4)	SD-PL (N = 4)
EF (%)	Baseline	65.2 ± 4.1	67 ± 3.1	64 ± 4	65.5 ± 1.9
	2 weeks	64.6 ± 4.9	66.9 ± 3.04	63.3 ± 3.4	63.7 ± 2.7
	4 weeks	57 ± 6.6	64.3 ± 6.4	62.6 ± 2.3	64.5 ± 3.9
	6 weeks	49 ± 5.4 ^a	62 ± 5.3	63 ± 3.8	66.4 ± 1.0
FS (%)	Baseline	32.7 ± 4.2	34.8 ± 4.2	31.2 ± 3.8	36.7 ± 0.5
	2 weeks	30 ± 4	30.2 ± 4	30.2 ± 1.5	35.2 ± 1.2
	4 weeks	26 ± 3.2	26.5 ± 3.2	28.5 ± 3.7	34.2 ± 2.2
	6 weeks	22.8 ± 4.8 ^a	30.2 ± 4.2	30.7 ± 3.5	34.4 ± 3.2
EDV (mL)	Baseline	0.41 ± 0.26	0.40 ± 0.17	0.44 ± 0.15	0.40 ± 0.07
	2 weeks	0.55 ± 0.21	0.41 ± 0.16	0.41 ± 0.1	0.4 ± 0.05
	4 weeks	0.81 ± 0.35	0.43 ± 0.09	0.41 ± 0.07	0.4 ± 0.06
	6 weeks	1.1 ± 0.68 ^a	0.43 ± 0.16	0.42 ± 0.03	0.42 ± 0.07

Abbreviations: EF, LV ejection fraction; EDV, end diastolic volume; FS, LV fractional shortening; LC, liposomal clodronate; LV, left ventricular; PBS, phosphate buffer saline; PL, PBS liposome; SD, Sprague Dawley.

^aP < .05 versus Ren-2 placebo and SD rats.

Table 4. Blood Pressure, Heart Weight, and Body Weight of LC- and PL-treated Rats

	Ren-2-LC (N = 7)	Ren-2-PL (N = 7)	SD-LC (N = 4)	SD-PL (N = 4)
BP (mm Hg)	253 ± 15 ^a	259 ± 10 ^a	131 ± 4.7	128 ± 10
BW (g)	314 ± 18	310 ± 23	314 ± 13.5	308 ± 8.9
HW/BW (%)	0.47 ± 0.04 ^a	0.50 ± 0.05 ^a	0.36 ± 0.03	0.35 ± 0.03

Abbreviations: BP, blood pressure; BW, body weight; HW/BW, heart weight to body weight ratio; LC, liposomal clodronate; PBS, phosphate buffer saline; PL, PBS liposomes; SD, Sprague Dawley.

^aP < .05 versus SD-LC and SD-PL.

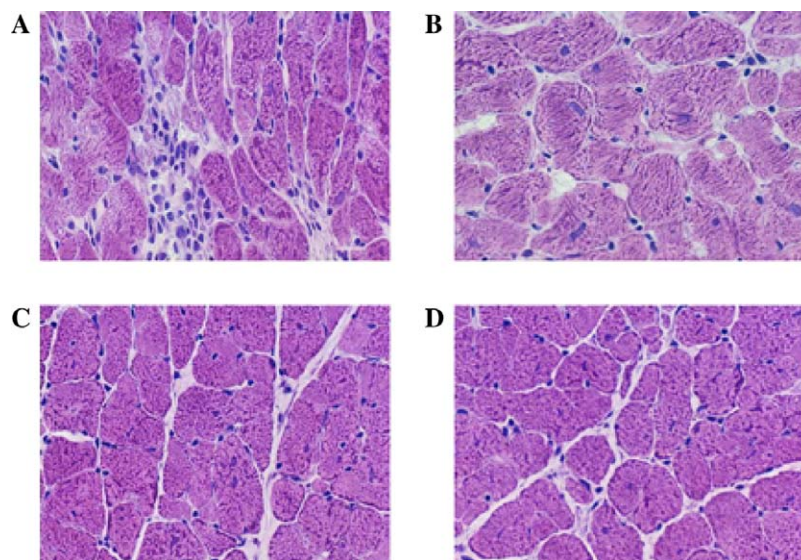


Figure 3. Histological characterization of the myocardial changes in LC-treated Ren-2 rats and controls. A, Hematoxylin-eosin stained myocardial sections showed areas of cardiomyocyte damage with mononuclear cellular infiltration in LC-treated Ren-2 rats. These changes were not remarkable in PL-treated Ren-2 (panel B), LC-treated SD (panel C), and PL-treated SD (panel D) rat hearts. LC = liposomal clodronate; PL = plain liposomes; SD = Sprague Dawley.

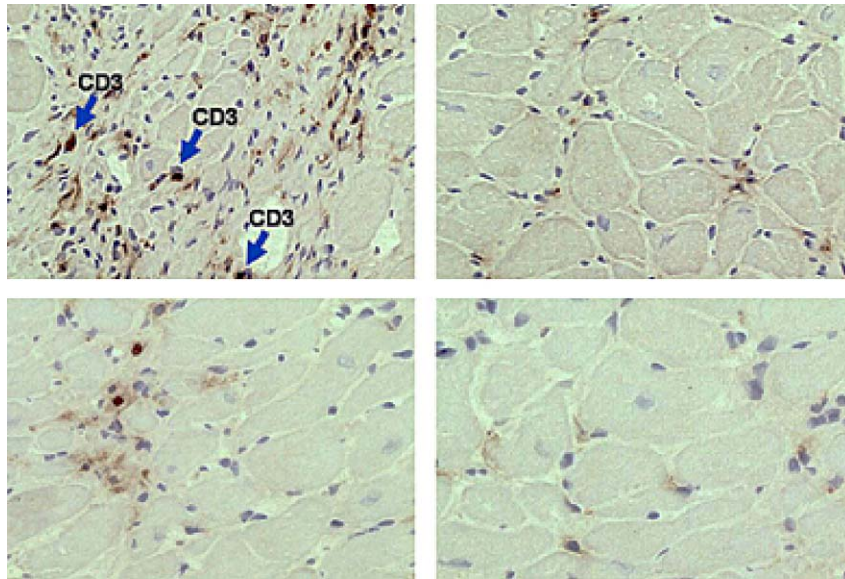


Figure 4. Demonstration of T-lymphocyte infiltration in LC-treated Ren-2 rat hearts. A, Anti-CD3 staining showing abundance of T lymphocytes in the hearts of Ren-2-rats treated with LC. B-D, Anti-CD3 staining in PL-treated Ren-2, LC-treated SD, and PL-treated SD rat hearts, respectively.

infiltrates of mononuclear cells were noted in the myocardium in LC-treated Ren-2 rat hearts. Morphologically, the infiltrated cells showed round nucleus with myocardial interstitial pattern of distribution (Figure 3A). Immunohistochemical analysis revealed that these round cells expressed CD3 (Figure 4) and CD4 antigen, suggesting that the macrophage-depleted Ren-2 rat hearts harbor increased CD4⁺ T-helper cells in the myocardium (Table 2).

Discussion

In contrast to our beginning hypothesis, the results of the current study suggest that the depletion of macrophages, in a model of hypertensive heart disease, results in the accelerated development of end-stage hypertensive cardiomyopathy and HF. The macrophage-depleted hearts showed infiltration with CD4⁺ T lymphocyte and multifocal loss of cardiomyocytes.

The possible role of macrophage infiltration in cardiomyopathy can be discussed as follows. First, minimal fibrosis was demonstrated and neither fibroblasts nor collagen deposition was observed. It is known that macrophage-related cytokines are associated with myofibroblast proliferation and

collagen production. Although development of interstitial fibrosis is an important component of cardiomyopathic process, it is reasonable to observe that complete abolition of fibrosis should be detrimental. In a postinfarction model, macrophage depletion has been reported to be associated with acute ventricular dilatation and early onset of HF.¹¹ It has also been demonstrated that intramyocardial injection of collagen prevents ventricular aneurysmal formation and HF.¹² Although these experiments highlight the importance of the collagen framework in healing from myocardial injury, neurohumoral antagonists (which have proven to be effective in prevention of remodeling and longevity) are known to reduce collagen deposition.¹³ It is therefore likely that both excessive collagen deposition as well as lack of collagen deposit is adversarial in myocardial remodeling. Second, cardiomyopathies are associated with increased circulating levels of various cytokines, including TNF- α , which are known to be detrimental for myocardial function.¹⁴ However, blocking of TNF- α has paradoxically resulted in worsening of experimental chronic Chagas disease-mediated cardiomyopathy,¹⁵ which is similar to our observations. Third, macrophage-depleted hearts showed abundant T-lymphocyte infiltration. The mechanisms for compensatory CD4⁺ T-cell infiltration in macrophage-depleted

hypertensive rats remain unclear. At least 4 distinct subsets of CD4⁺ T cells have been recognized including Th1, Th2, Treg, and Th17 cells. Treg appear to play a protective role¹⁶ and Th2 cells play a role in antibody-mediated response; as such CD4⁺ T cells in our study are unlikely to be Treg or Th2 cells. Therefore, we assume that the CD4⁺ T-cell infiltrates may be composed of either Th1 cells, Th17 cells, or both. Th17 cells secrete a number of proinflammatory cytokines, including interleukin (IL)-17, and have shown to play an important role in autoimmunity and inflammation.¹⁷⁻¹⁹ It has been shown that IL-17 mediates experimental autoimmune cardiomyopathy in t-bet knock out mice (t-bet favors Th1 development), suggesting a role of IL-17 in cardiomyopathy.²⁰ Taken together, sustained depletion of macrophages could contribute to the deterioration of cardiac function because of the loss of the beneficial aspects of macrophage function and/or deleterious effects of enhanced CD4⁺ T-cell infiltration.

Conclusions

The results of the current study show a potentially important role of macrophages in ventricular remodeling in hypertension. Macrophage activation may be important in repair processes and debris clearance and hence depletion of macrophages may not necessarily be an effective strategy in prevention of HF. However, larger studies may be needed to evaluate the role of modest modulation of macrophage function.

Acknowledgments

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